



Reactivation of Creatine Kinase by Dithiothreitol Prior to Use in an *In Vitro* Translation Extract

Daniel Favre and Beat Muellhaupt

Division Gastroenterology and Hepatology, Department of Internal Medicine, University Hospital of Zurich, CH-Zurich

Summary

Background: *In vitro* protein synthesis on exogenous messenger ribonucleic acids can be performed in various systems including cytoplasmic extract from eukaryotic cells, rabbit reticulocyte lysate and wheat germ extract. For optimal translation, an energy regeneration system based on creatine phosphate and creatine kinase is commonly employed for the regeneration of the endogenous adenosine triphosphate pools. Creatine kinase purchased from various commercial suppliers can be partially oxidised. Oxidised creatine kinase is not biologically active and might not allow the efficient initiation of translation of exogenous mRNAs in eukaryotic cell extracts *in vitro*.

Results: We successfully used dithiothreitol to reduce and therefore reactivate commercially available creatine kinase. When employed in cytoplasmic extracts obtained from eukaryotic cells grown in monolayers, the reactivated creatine kinase restored translation of the exogenous mRNAs.

Conclusion: Lyophilised creatine kinase obtained from commercial suppliers can be purchased as an oxidised monomer. The reactivation of creatine kinase using a reducing agent such as dithiothreitol restores the biological activity of this enzyme. This procedure might therefore be extended to various other *in vitro* conditions and biological systems in which the maintenance of an efficient ATP-regenerating system is critical.

Zusammenfassung: Reaktivierung der Kreatinkinase durch Dithiothreitol vor Verwendung in einem *in vitro* Translations-Extrakt

Hintergrund: Die *in vitro* Proteinsynthese mit exogener mRNA kann in verschiedenen Systemen wie z. B. zytoplasmatischen Extrakten von eukariotischen Zellen, Retikulozyten-Lysaten von Kaninchen und Weizenextrakten durchgeführt werden. Für eine optimale Translation wird in der Regel ein Energieregenerationssystem mit Kreatinphosphat und Kreatinkinase verwendet, um eine Regenerierung des endogenen Adenosintriphosphat-Pools zu erreichen. Kreatinkinase, die von verschiedenen kommerziellen Anbietern erhältlich ist, kann partiell oxidiert sein. Die oxidierte Kreatinkinase ist aber biologisch nicht aktiv und kann dafür verantwortlich sein, dass die effiziente Translations-Initiation von exogener mRNA in eukariotischen Zell-extrakten *in vitro* nicht möglich ist.

Ergebnisse: Wir haben erfolgreich Dithiothreitol benutzt, um partiell oxidierte Kreatinkinase zu reduzieren und dadurch ihre biologische Aktivität zu regenerieren. Die reaktivierte Kreatinkinase ermöglichte die effiziente Translation von exogener mRNA in zytoplasmatischen Extrakten von eukariotischen Zellen.

Konklusion: Lyophilisierte Kreatinkinase, die von kommerziellen Anbietern erworben wird, kann in oxidiert Form vorliegen. Diese kann durch eine reduzierende Substanz wie z. B. Dithiothreitol reduziert werden, wodurch die biologische Aktivität der Kreatinkinase wieder hergestellt werden kann. Dieses Vorgehen könnte auch für andere *in vitro* Systeme, bei denen effiziente ATP Regeneration wichtig ist, von Bedeutung sein.

Keywords: translation, protein synthesis, *in vitro*, creatine kinase, reactivation, ATP regeneration, dithiothreitol, N-acetylcysteine, hepatitis B virus, HBV, polymerase

1 Background

Rabbit reticulocyte lysate, Krebs ascites fluid and wheat germ extract have received the most attention as cell-free translation systems that most closely resemble the *in vivo* protein machinery

(Clemens, 1979; Pelham and Jackson, 1976). Since they are not fully representative of eukaryotic cells in the way they regulate translation, eukaryotic cells grown as monolayers can be prepared and employed for the generation of a translational extract for the translation of

exogenous messenger ribonucleic acids (mRNAs) (Brown et al., 1983; Favre and Trepo, 2001; Skup and Millward, 1977).

Regeneration of adenosine triphosphate (ATP) is essential in various biochemical processes. Commercially available creatine kinase (CK; adenosine 5'-triphosphate-creatine phosphotransferase, EC 2.7.3.2) from rabbit muscle is

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usually employed *in vitro* for the regeneration of the endogenous ATP pools (Clemens, 1979). It is usually purchased as a lyophilised stock that is resuspended in water containing 50% glycerol for further use and long-term storage in the freezer. This enzyme catalyses the reversible transfer of a phosphoryl group from creatine phosphate to adenosine diphosphate to regenerate ATP under physiological pH. It is active as a dimer with two reduced identical subunits of 42 kDa that are not covalently bound (Dawson et al., 1967). It contains four SH groups of cysteine residues per subunit; only the SH groups on residues Cys-73 and Cys-282 are essential for the catalytic activity of CK (Hurne et al., 2000; Wang and Seeger, 1992). Various sulfhydryl agents, some chelating agents and adenosine phosphate compounds, adenosine, Cl⁻, or SO₄⁻ are known to inhibit creatine kinase (Kuby and Noltman, 1962), whereas Mg²⁺, Ca²⁺ and Mn²⁺ are known to preserve the biological activity of CK (Gercken and Doring, 1974). Thus, the sustained biological activity of CK might be critical for the generation of cell extracts for *in vitro* translation. Indeed, we have previously shown that the addition of sucrose during the preparation and storage of cell extracts could to some extent prevent the loss of the biological activity of the creatine kinase (Favre and Trepo, 2001).

It has been shown that the biological activity of denatured CK could be restored after treatment with dithiothreitol (DTT) or glutathione (Hurne et al., 2000). It was suggested that there is a strong relationship between the oxidised and reduced forms of the monomers of CK and their enzymatic activity. The presence of more than 50% of the 37-kDa oxidised form abolished CK's biological activity (Hurne et al., 2000). Since the reducing activity of DTT on CK and the consequences for its activity have never been directly examined in downstream *in vitro* applications such as *in vitro* translation, experiments were performed using reactivated creatine kinase added to *in vitro* translational extracts obtained from eukaryotic cells grown as monolayers (Favre and Trepo, 2001).

2 Methods

2.1 Analysis of creatine kinase activity without prior reactivation of the enzyme

The creatine kinase activity was determined in a coupled enzyme system utilising pyruvate kinase and lactate dehydrogenase, as described elsewhere (Tanzer, 1959; Worthington Biochemical Corporation). One unit is defined as the conversion of one micromole of creatine to creatine phosphate per minute at 25°C and pH 8.9 under the specified conditions.

2.2 Analysis of the creatine kinase activity with prior reactivation of the enzyme

A commercial *in vitro* test for the quantitative determination of liquid CK in human serum or plasma (Roche) was employed. In this test, equimolar quantities of NADPH and ATP are formed and the formation of NADPH is directly proportional to the activity of CK. One unit is defined as described above. This assay is routinely performed according to the International Federation of Clinical Chemistry (IFCC) for the detection of putative myocardial infarction or myocarditis. In this test, the enzyme is reactivated by the presence of N-acetyl-cysteine (NAC; 24.6 mM) contained in the commercial assay kit.

2.3 Treatment of creatine kinase with dithiothreitol or N-acetyl-cysteine

Creatine kinase from rabbit muscle was purchased as a lyophilised stock from commercial sources. In order to render the enzyme biologically active by reduction with a reducing agent (Hurne et al., 2000), the enzyme was resuspended at 100 mg per ml in 40 mM Tris [pH 7.6] containing either 20 mM DTT or 20 mM NAC. Reactivation was performed for 30 min at room temperature. The enzyme was then stored between -15°C and -20°C at 5 mg per ml as a 50% glycerol stock. Enzymatic activity was determined as for the non-reactivated CK, as described above.

2.4 *In vitro* translation

Baby hamster kidney (BHK) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with

antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) and 10% heat-inactivated foetal calf serum (FCS). The human hepatocyte cell line Huh-7 (Nakabayashi et al., 1982) was grown in Eagle's Modified Essential Medium supplemented with 10% FCS, 1% sodium pyruvate, 2 mM glutamine and antibiotics. *In vitro* transcription of capped messenger RNAs (mRNAs) on linearised plasmids was performed essentially as described (Svitkin et al., 1994). Translational extract was prepared as described elsewhere (Brown et al., 1983; Favre and Trepo, 2001). In order to allow efficient incorporation of radiolabelled amino acids such as methionine or cysteine in the *in vitro* translated polypeptides, the cells were preincubated for 30 min at 37°C under 5% CO₂ with DMEM lacking methionine or lacking both methionine and cysteine (Sigma). The cells were then washed with washing buffer (20 mM Hepes [pH 7.4], 33 mM NH₄Cl, 7 mM KCl, 150 mM sucrose) and thereafter the cytoplasmic membranes of the cells were lysed for 90 s using 100 µg lysolecithin, palmitoyl (Avanti Polar Lipids; stock at 10 mg/ml in chloroform/methanol (1:1) at -20°C) per ml in washing buffer, on ice. Following the complete removal of lysolecithin from the Petri dish, the cells were scraped into 200 µl extraction buffer containing 100 mM Hepes-KOH [pH 7.4], 120 mM potassium acetate [pH 7.4], 2.5 mM magnesium acetate, 1 mM DTT, 2.5 mM ATP, 1 mM GTP, 100 µM S-adenosyl-methionine, 1 mM spermidine, 20 mM creatine phosphate, 40 units of DTT-treated creatine phosphokinase (Sigma) per ml, 40 µM of each essential amino acid except methionine, or except methionine plus cysteine (Promega) and 100 mM sucrose. The cells were then passed ten times through a 25-gauge needle, and the lysate was centrifuged at 4°C and 100 x g for 2 min. The supernatant was collected to prepare the mRNA-dependent lysate; for this, the endogenous mRNAs were hydrolysed by incubating the lysate at 20°C for 7 to 10 min in the presence of 10 units of micrococcal nuclease (P-L Biotechnology) per ml and 1 mM CaCl₂. The enzyme was inhibited by adding 2.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-

tetraacetic acid [pH 7] (EGTA). The volume of micrococcal nuclease plus CaCl_2 plus EGTA that was added represented 4% of the final volume of the translational extract. A Petri dish [10 cm diameter] provides 0.2 ml of translational extract obtained from about 10^7 cells. The latter extract could be used immediately for translation of exogenously added mRNAs, or could be frozen at -70°C until further use. After thawing, 200 μl of extract were supplemented with 1 μl of 5 mg/ml CK reactivated with dithiothreitol, as described above. *In vitro* translation in 20 μl was carried out by mixing 15 μl of the micrococcal nuclease-treated extract in which [^{35}S]methionine or [^{35}S]methionine/cysteine (translation grade; >1000 Ci/mmol) and exogenous mRNA (final concentration: 5 to 10 μg per ml) have been added. Translation reactions were carried out at 30°C for 60 min. The reactions were stopped by addition of 2X sodium dodecyl sulphate (SDS)-sample buffer followed by boiling for 3 min. The radiolabelled polypeptides were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) followed by autoradiography.

3 Results

3.1 Reactivation of creatine kinase with dithiothreitol

The biological activity of CK was initially assessed by employing a commercial assay kit containing N-acetyl-cysteine (NAC) and by following the supplier's instructions. It turned out that CK that was not previously treated with DTT was biologically active, with values up to 15 U/mg protein, independent of the supplier (Tab. 1). In contrast, when CK activity was measured in the absence of a reducing agent such as DTT or NAC, no biological activity could be measured. The biological activity of CK could be restored by reactivation with DTT or NAC. The reactivated CK possessed enzymatic activity ranging between 0.12 and 1.5 U/mg of protein in this assay.

Interestingly, when the creatine kinase purchased from commercial sources was analysed by SDS-PAGE in the absence of any reducing agent such as DTT or 2-mercaptoethanol, 50% or more of the

enzyme was found to be in the oxidised state, as shown by the migration of the native 37-kDa oxidised polypeptide (Fig. 1, lanes 1 and 3). In contrast, when the enzyme was previously treated with DTT, more than 95% of the enzyme was found to be in the biologically active, reduced state of 42 kDa (lanes 2 and 4). Therefore, reactivation of oxidised CK with DTT might be a prerequisite for the restoration of the enzymatic activity, namely the regeneration of ATP pools

by hydrolysis of creatine phosphate, when employed in an *in vitro* translation system.

3.2 *In vitro* translation of exogenous mRNAs in eukaryotic cell extracts using reactivated creatine kinase

In order to analyse the enzymatic activity of CK reactivated or not with DTT in an *in vitro* assay, translation reactions were performed with micrococcal nucle-

Tab. 1: Reactivation of the enzymatic activity of creatine kinase with reducing agents

^aCreatine kinase activity measured by employing a commercial assay kit (Roche) containing N-acetyl-cysteine (NAC). ^bCreatine kinase activity measured by employing a laboratory procedure devoid of reducing agents, without or with prior treatment of the CK with dithiothreitol (DTT) or N-acetyl-cysteine (NAC). ^cEnzymatic activity of CK: -, no enzymatic activity, ++ and +++, efficient creatine kinase activity.

Reactivation of CK	Commercial assay kit ^a	Laboratory procedure ^b		
	NAC	∅	DTT	NAC
Creatine kinase activity ^c	+++	-	++	++

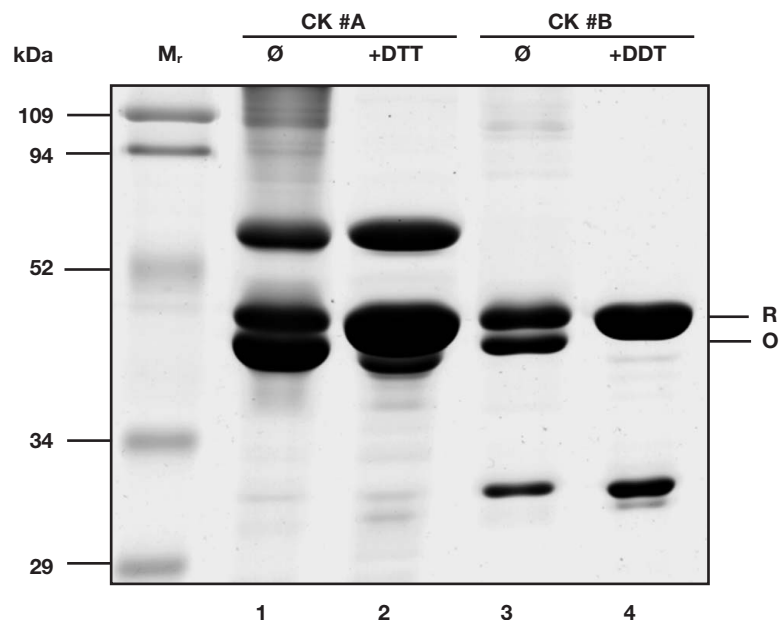


Fig. 1: Reactivation of creatine kinase for further use in translation *in vitro*

Creatine kinase purchased from two commercial sources (CK #A and CK #B) analysed by 12% SDS-PAGE under non-reducing conditions followed by Coomassie Blue staining. Both the oxidized (O) form of 37 kDa and the reduced (R) form of 42 kDa of the CK monomer are visible (lanes 1 and 3). Creatine kinase reactivated with the reducing agent dithiothreitol: the biologically active, reduced form of 42 kDa of the monomer is visible (lanes 2 and 4). Measurements of enzymatic activities are presented in Table 1. M_r, relative molecular mass in kDa.



ase-treated extracts programmed with various exogenously added mRNAs, such as hepatitis B virus polymerase and duck hepatitis B virus polymerase mRNA. *De novo* translation could be achieved on these mRNAs when the translational extracts obtained from both BHK (Fig. 2, A and B) and Huh-7 (C) cells were supplied with reduced CK that had been reactivated with DTT (lanes 5, 6 and 8). As expected, major polypeptides of 94 and 81 kDa were obtained by translation of the HBV polymerase mRNA. The second polypeptide is attributed to initiation at a second AUG codon; a minor polypeptide of 40 kDa was also translated (lane 5), as also shown elsewhere (Li and Tyrrell, 1999; Seifer et al., 1998). Similarly, the expected polypeptide dimer of 85 and 90 kDa was obtained by *in vitro* translation of the duck HBV polymerase mRNA (lane 6) (Pollack and Ganem, 1994). In contrast, when the extracts were supplemented with creatine kinase that was not previously reactivated (and therefore contained this enzyme mostly in its oxidised, biologically inactive form), translation of the exogenously added mRNAs did not occur in the BHK cell extract (lanes 2 and 3), even though the translational extract already contained DTT (1 mM). Additional, unrelated polypeptide bands of about 100 to 110 kDa were also visible. Similar results were obtained when translational extracts from Huh-7 cells were employed (data not shown).

4 Discussion

We previously reported on *in vitro* translation assays with cytoplasmic extracts obtained from eukaryotic cells grown as monolayers (Favre and Trepo, 2001). In those initial experiments, creatine kinase from a commercial source was directly resuspended in 50% glycerol and the use of 100 mM sucrose in the translational extract was found to be sufficient to keep the enzyme biologically active. However, since then we have repeatedly observed that the use of CK from commercial sources did not always allow the efficient translation of exogenous mRNAs when employed without treatment other than

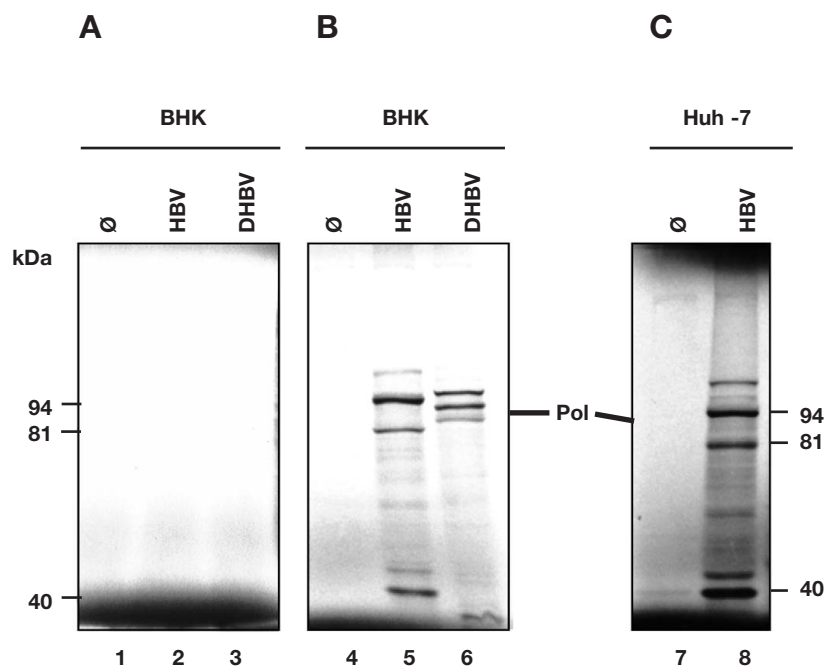


Fig. 2: Translation of exogenous mRNAs in cytoplasmic extracts from eukaryotic cells

The baby hamster kidney (BHK) cell line (A, B) and the human hepatocyte cell line Huh-7 (C) were employed for the generation of extracts that served for the translation of *in vitro* transcribed, exogenous mRNAs coding for either the hepatitis B virus (HBV) polymerase (lanes 2 and 5) or the duck HBV (DHBV) polymerase (lanes 3, 6 and 8) analysed by 10% SDS-PAGE followed by autoradiography. Prior to *in vitro* translation, the extract was pre-treated with micrococcal nuclease in order to hydrolyse the endogenous mRNAs, and thereafter supplemented with creatine kinase that was either not reactivated (A) or was reactivated (B, C) with dithiothreitol. As a control, translation reactions were performed without addition of exogenous mRNA (lanes 1, 3 and 7). *M_r*, relative molecular mass in kDa.

resuspension in 50% glycerol and storage in the presence of 100 mM sucrose (data not shown).

Creatine kinase is very hygroscopic and is usually purchased with a humidity adsorbent. Therefore, it is possible that a substantial portion of the enzyme is present in the oxidised, biologically inactive state. This CK might therefore not be effective in regenerating the endogenous ATP pools under physiological conditions. Surprisingly, neither the presence of ATP (2.5 mM) nor the presence of DTT (1 mM) in the reaction buffer was sufficient to enable translation of exogenous mRNAs. Therefore, we examined

several parameters, such as the pH and the biological activity of the various compounds employed in the translation reaction (referred to as “extraction buffer”) (Favre and Trepo, 2001) in order to circumvent this inhibition and to restore the translation on the exogenously added mRNAs.

We finally found that the oxidation status of the lyophilised CK was the crucial parameter (Fig. 1). When commercial CK was directly resuspended and stored in 50% glycerol containing 100 mM sucrose, 50 to 70% of the monomer was oxidised and migrated with an apparent molecular weight of 37 kDa in SDS-

PAGE under non-reducing conditions (lanes 1 and 3). When employed in *in vitro* reactions to regenerate endogenous ATP pools, no translation of the exogenous mRNA was obtained (Fig. 2A). We hypothesised that a higher amount of reduced CK was needed for efficient *in vitro* translation. To reduce the CK, the enzyme was pretreated with DTT under physiological pH, as described (Park et al., 2000). After treatment and storage in 50% glycerol containing 100 mM sucrose, a single major polypeptide band of 43 kDa was visible in SDS-PAGE (lane 2). This renatured CK was then employed in *in vitro* translation extracts that had been pretreated with micrococcal nuclease in order to hydrolyse the endogenous mRNAs. The results showed that translation of exogenous mRNAs was efficiently restored. The expected polypeptide sizes 94 and 81 kDa, and 90 and 85 kDa resulting from *in vitro* translation of the HBV and DHBV polymerase mRNAs, respectively (Fig. 2) (Li and Tyrrell, 1999; Pollack and Ganem, 1994) were obtained.

The use of a commercial assay already containing a reducing agent such as N-acetyl-cysteine in order to measure CK activity is not recommended when lyophilised creatine kinase obtained from commercial sources is employed. Indeed, the presence of NAC in the reaction solution reactivates the oxidised, biologically inactive CK (Horder et al., 1991; Klauke et al., 1993). Therefore, when an oxidised, commercial CK is analysed by this method, the enzymatic activity will always be optimal and does therefore not represent the true biological activity of the enzyme.

A subtle relationship might exist between the oxidised and reduced forms of the CK monomers and the enzymatic activity (Hurne et al., 2000). ATP, as a key element in the energy flow of biological reactions, can be regenerated with the use of biologically active creatine kinase. The reactivation of the creatine kinase might therefore not be crucial solely for *in vitro* translation, but should be extended to other procedures in which this enzyme is employed for regeneration of endogenous ATP pools. This reactivation might also be performed using various

additives (Lott and Heinz, 1982; Nealon et al., 1981; Penny and Jennings, 1988) other than dithiothreitol.

In conclusion, the procedure described here can be used for various cell lines grown as monolayers and can replace the use of the rabbit reticulocyte lysate for translation of exogenous messenger RNAs *in vitro*.

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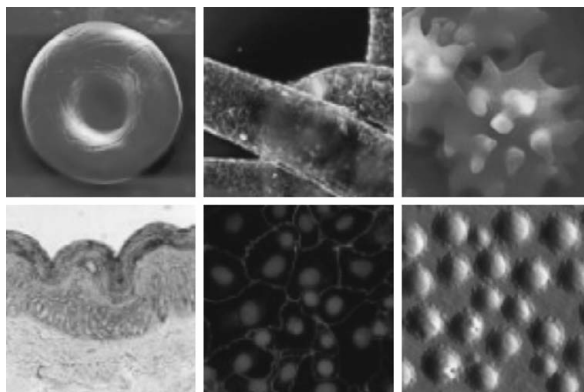
Correspondence to

Dr. Daniel Favre
Division Gastroenterology and
Hepatology
Department of Internal Medicine
University Hospital of Zurich
Raemistrasse 100
8091 Zurich Switzerland
e-mail: Daniel.Favre@usz.ch

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